Associations between Virulence Factors of Shiga Toxin-Producing *Escherichia coli* and Disease in Humans

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Associations between known or putative virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans were investigated. Univariate analysis and multivariate logistic regression analysis of a set of 237 isolates from 118 serotypes showed significant associations between the presence of genes for intimin (*eae*) and Shiga toxin 2 (*stx*$_2$) and isolates from serotypes reported in humans. Similar associations were found with isolates from serotypes reported in hemorrhagic colitis and hemolytic-uremic syndrome. The enterohemorrhagic *E. coli* (EHEC) hemolysin gene was significantly associated with isolates from serotypes found in severe diseases in univariate analysis but not in multivariate logistic regression models. A strong association between the intimin and EHEC-hemolysin genes may explain the lack of statistical significance of EHEC hemolysin in these multivariate models, but a true lack of biological significance of the hemolysin in humans or in disease cannot be excluded. This result warrants further investigations of this topic. Multivariate analysis revealed an interaction between the *eae* and *stx*$_2$ genes, thus supporting the hypothesis of the synergism between the adhesin intimin and Shiga toxin 2. A strong statistical association was observed between the *stx*$_2$ gene and severity of disease for a set of 112 human isolates from eight major serotypes. A comparison of 77 isolates of bovine origin and 91 human isolates belonging to six major serotypes showed significant associations of the genes for Shiga toxin 1 and EspP protease with bovine isolates and an increased adherence on HEp-2 cell cultures for human isolates, particularly from diarrheic patients and healthy persons.

Shiga toxin-producing *Escherichia coli* strains (STEC) were first implicated in disease in the early 1980s by their association with hemolytic-uremic syndrome (HUS) and hemorrhagic colitis (HC) (16, 27). STEC have subsequently been associated with uncomplicated diarrhea (23) and have been isolated from stools of healthy individuals. STEC are now considered a major cause of disease in developed countries (10, 17). HC usually begins with abdominal cramps and diarrhea, followed by bloody diarrhea. HUS patients present with acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, often following a prodromal diarrhea. HC and HUS are severe diseases which frequently require hospitalization, and HUS may be fatal in up to 5% of cases. STEC infections are mainly food borne, and bovine feces are the main source of food contamination by this organism (10). A large variety of STEC serotypes have been implicated in human disease, but some STEC serotypes found in cattle or in food have never or only very rarely been associated with severe human disease. These apparent differences in STEC serotype frequencies may, in part, be due to methodological issues, but differences in the ability of STEC strains to cause disease are also likely contributors.

Based on in vitro and animal model studies, several virulence factors have been described in STEC, the major one being Shiga toxins (11). Two main categories of Shiga toxins have been distinguished. *E. coli* Shiga toxin 1 (Stx1) is almost identical to the Shiga toxin of *Shigella dysenteriae* in amino acid sequence and cannot be distinguished from it serologically,

whereas Shiga toxin 2 (Stx2) is less related to the Shiga toxin of *Shigella* and is not neutralized by antibodies to either Stx1 or Shiga toxin from *S. dysenteriae* (21, 35). As is the case with enteropathogenic *E. coli*, some STEC strains can tightly attach to epithelial cells of the intestine through an adhesin called intimin. Such strains induce in the underlying cells profound structural modifications called attaching and effacing lesions. The genes related to these lesions, including the *eae* (for *E. coli* attaching and effacing) gene, which encodes intimin, are clustered in a pathogenicity island named the locus for enterocyte effacement (LEE [19]). Recently, Schmidt and collaborators reported the genetic analysis of a new plasmid-encoded hemolysin of STEC called enterohemorrhagic *E. coli* hemolysin (EHEC hemolysin; *ehxA* gene), which seemed to be associated with severe clinical disease in humans (31, 32). A protease (EspP), encoded by the same plasmid as EHEC hemolysin, has also recently been described in some STEC serotypes and has been suggested as an additional virulence factor of STEC (5). There is actually no experimental proof for the role of EHEC hemolysin and EspP in the virulence of STEC. They are therefore only putative virulence factors, but for the sake of simplicity, they will be included with the other virulence factors for the remainder of the discussion.

Previous studies have shown a large diversity in the distribution of virulence factors among STEC strains (1, 3, 15, 41). Associations have been suggested between the presence of some of these factors in STEC and their virulence (24, 29, 30, 32). However, these studies were often relatively small scale or examined the distribution of each virulence factor separately, without accounting for possible associations between virulence factors and without considering the rest of the genome of the bacterial pathogen. In the present study, the distribution of virulence factors in an international collection of STEC iso-

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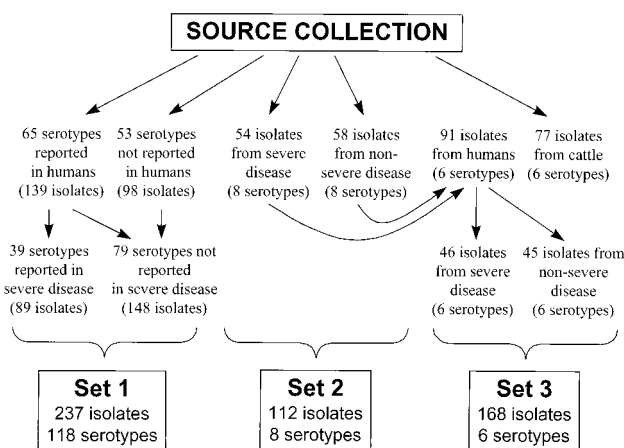
FIG. 1. Graphical representation of sampling strategy used to obtain sets 1 to 3 of STEC isolates.

lates representing a broad spectrum of serotypes from various sources was determined and analyzed by methods which account for these possible influences. The first aim of the study was to determine associations between virulence factors and STEC disease in humans, based on classification of STEC isolates by serotypes reported or not reported in the literature to have been isolated from humans. Multivariate analysis was used to control for the confounding effects of other virulence factors and of the genomic background of the isolates by using serotype as a proxy. The second aim was to examine the diversity of virulence factors in serotypes most frequently associated with disease and to detect associations between any of these factors and the severity of disease in the actual patients from whom the isolates were recovered. The last aim of this study was to compare bovine and human STEC populations of the major serotypes involved in human disease to test whether human STEC from these serotypes that are most commonly isolated from patients with disease form a different population than the bovine STEC population of the same serotypes.

MATERIALS AND METHODS

STEC isolates. Three different sets of STEC isolates were used for the present study (Fig. 1). The first set comprises 237 STEC isolates of 118 serotypes originating from humans ($n = 60$), animals ($n = 159$), and food ($n = 18$) that were selected from a larger collection of STEC isolates deposited at the Health of Animals Laboratory, Health Canada, Guelph, Ontario, Canada. Stratified random sampling (strata are equivalent to serotypes) was used for the selection in order to represent all the serotypes available in the collection. The number of isolates per serotype was limited to a maximum of three, and for those serotypes with less than three isolates in the collection, all were used. The source collection is the fruit of a long-term effort to collect representative STEC isolates from

human and nonhuman sources of diverse geographic origin. Based on an extensive review of the literature, the isolates were classified into four categories (Fig. 1). The first category within set 1 (listed below) comprised 139 isolates belonging to 65 serotypes previously reported in humans (O1:H20, O2:H5, O2:H6, O2:H27, O2:H29, O5:H− (nonmotile isolates) O6:H−, O7:H4, O8:H14, O15:H27, O15:H−, O22:H8, O22:H16, O26:H11, O26:H−, O38:H21, O45:H2, O48:H21, O55:H7, O55:H9, O75:H1, O76:H19, O80:H−, O82:H8, O84:H2, O89:H−, O91:H10, O91:H14, O91:H21, O91:H−, O98:H−, O103:H2, O111:H8, O111:H−, O112:H2, O113:H4, O113:H7, O113:H21, O114:H4, O115:H18, O117:H4, O118:H12, O118:H16, O118:H30, O119:H6, O119:H−, O121:H19, O126:H8, O126:H21, O128:H2, O128:H−, O132:H−, O145:H−, O146:H8, O146:H21, O153:H25, O157:H7, O157:H−, O163:H19, O163:H−, O165:H25, O165:H−, O171:H2, O172:H−, and OX3:H21). The second category comprised 98 isolates of 53 serotypes not previously reported in humans (O2:H39, O2:H−, O5:H11, O6:H10, O6:H34, O8:H8, O8:H9, O8:H16, O8:H19, O8:H35, O15:H7, O22:H2, O39:H49, O40:H8, O43:H2, O46:H38, O46:H−, O49:H−, O69:H11, O76:H25, O77:H39, O84:H−, O85:H−, O88:H25, O91:H7, O98:H25, O110:H8, O111:H11, O113:H−, O115:H8, O116:H21, O118:H−, O119:H5, O119:H25, O121:H7, O126:H27, O128:H35, O130:H38, O132:H18, O136:H12, O136:H16, O136:H−, O139:H19, O142:H38, O145:H8, O153:H21, O153:H31, O156:H7, O156:H8, O156:H25, O156:H−, O163:H2, and O168:H8). The third category is a subset of the first one and comprised 89 isolates belonging to 39 serotypes clearly identified in the literature as associated with HUS and HC (underlined in the first list above). The fourth category of isolates within set 1 corresponds to the remaining 148 isolates of 79 serotypes not previously reported in severe human disease. No significant difference between categories in terms of mean number of isolates per serotype was detected by a z test. The overall mean number of isolates per serotype was 2.008. This supports our attempt to control for serotype confounding in set 1 at the sampling level.

The second set (Fig. 1 and Table 1) comprises 112 epidemiologically unrelated isolates of human origin belonging to eight serotypes often associated with human disease. These isolates represent all the human isolates of these serotypes from the STEC collections deposited at the Health of Animals Laboratory, Health Canada, Guelph, for which suitable clinical information was available. They originate from Belgium ($n = 53$), Germany ($n = 17$), Switzerland ($n = 16$), the United States ($n = 14$), Canada ($n = 6$), Australia ($n = 3$), and Denmark ($n = 3$). Based on clinical information available from the donors (Table 1), these isolates were further classified into two categories. The first category (nonsevere disease) comprises isolates from healthy persons and from patients with uncomplicated nonbloody diarrhea. The second category (severe disease) comprises isolates from patients with bloody diarrhea or from patients with clinical signs of HUS.

The third set of isolates (Fig. 1 and Table 1) comprises all the human isolates from six of the eight serotypes of set 2 and identical numbers of randomly chosen isolates of bovine origin of the same serotypes. For serotypes for which there were fewer isolates of bovine origin than of human origin, all the bovine isolates were used.

Detection of stx_1, stx_2, eae, $ehxA$, and $espP$. All the isolates were examined for the presence of the stx_1 and stx_2 genes by PCR under the conditions described by Pollard and collaborators (25) except for three isolates for which the Cangene primers and conditions were used (26). The STEC strains EC910004 (serotype O46:H38; bovine origin) and 4304 (serotype O157:H7; human origin) served as positive controls, and the enteropathogenic *E. coli* strain 2348/69 served as a negative control for this test. The presence of *eae* was detected by PCR under the conditions described by Sandhu and coworkers (30) and was confirmed by dot blot hybridization when necessary. Strains 4304 and JM109 (43) served as positive and negative controls, respectively. For the dot blot hybridization, the probe consisted of the digoxigenin-labeled PCR product of strain 4304 produced with the DIG DNA labeling and detection kit (Boehringer, Mannheim, Germany). Cell lysates were obtained by resuspending the cells of a 500-μl overnight culture in Luria-Bertani broth in 100 μl of 0.4 M NaOH and heating it for 30 min at 80°C. One microliter of cell lysate was blotted on a Hybond membrane (Amersham Life Science, Little Chalfont, England) and bound by UV cross-linking.

TABLE 1. Numbers of STEC isolates of human and bovine origin in strain sets 2 and 3 classified by serotype

Isolate source[a]	No. of isolates								
	O26:H11	O26:H−	O103:H2	O111:H8	O111:H−	O145:H−	O157:H7	O157:H−	Total
NS	5	0	1	0	0	1	0	3	10
D	5	7	9	1	10	1	12	3	48
BD	1	2	5	1	1	1	13	0	24
HUS	4	2	1	0	4	3	12	4	30
Total human	15[b]	11	16[b]	2[b]	15[b]	6[b]	37[b]	10	112
Bovine	8[b]	0	16[b]	2[b]	13[b]	6[b]	32[b]	0	77

[a] NS, STEC isolates from humans with no symptoms; D, isolates from humans with uncomplicated diarrhea; BD, isolates from humans with bloody diarrhea; HUS, isolates from humans with hemolytic-uremic syndrome. All of the human isolates were used for set 2.

[b] Isolate used for set 3.

TABLE 2. Overall distribution of *ehxA*, *espP*, *eae*, *stx₁*, and *stx₂* in STEC isolates from serotypes which are reportedly not found in humans, from serotypes found in humans, and from serotypes clearly associated with severe disease in humans (set 1)

Gene	Distribution[a] in serotypes:			
	Total	Not from humans	From humans	From severe disease
ehxA	146 (61.6)	59 (60.2)	87 (62.6; 0.7100)	64 (71.9; 0.0114)
espP	148 (62.4)	60 (61.2)	88 (63.3; 0.7441)	55 (61.8; 0.8728)
eae	77 (32.5)	23 (23.5)	54 (38.8; 0.0128)	43 (48.3; 0.0001)
stx₁	148 (62.4)	72 (73.5)	76 (54.7; 0.0033)	46 (53.9; 0.0358)
stx₂	134 (56.5)	42 (42.9)	92 (66.2; 0.0004)	63 (70.8; 0.0006)
No. of isolates	237	98	139	89
No. of serotypes	118	53	65	39

[a] Of the total 237 isolates of set 1, 159 were of animal origin, 18 were of food origin, and 60 were of human origin. The numbers in the columns represent numbers of isolates. The first number in parentheses indicates the percentage of the total category positive for the respective characteristic. The second number in parentheses for the fourth column indicates the *P* value for chi-square tests comparing isolates from serotypes found in humans with those not found in humans. The second number in parentheses in the last column indicates the *P* value for chi-square tests comparing isolates from serotypes associated with severe disease to all the others.

Hybridization was done following standard protocols (28) with stringent washing at 65°C in 0.2× SSC (20× SSC is 3 M NaCl plus 0.3 M sodium citrate, pH 7.0). Probe that remained bound to homologous sequences was detected with the DIG DNA labeling and detection kit following the supplier's instructions. The presence of *ehxA* was detected by PCR following the method of Sandhu and collaborators (29). Strains 4304 and 2348/69 served as positive and negative controls, respectively. Expression of the hemolytic phenotype was detected by incubating isolates overnight on washed sheep erythrocyte plates (2). When PCR and phenotype results were contradictory, dot blot hybridization of plasmid DNA was used to confirm the results with a digoxigenin-labeled probe made of the *ehxA* PCR product of strain 4304 as described by Boerlin and collaborators (4). Plasmid preparations were made by the alkaline lysis method (28) with one phenol-chloroform extraction, and 1 μl of each preparation was blotted onto Hybond membrane and bound by UV cross-linking. Hybridization and detection were done under the same conditions as for *eae*. For detection of *espP*, the same dot blot plasmid hybridization method was used as for *ehxA*. The digoxigenin-labeled probe consisted of a PCR product from strain 4304 covering the whole *espP* coding sequence. Strains 4304 and 2348/69 served as positive and negative controls, respectively. In case of questionable results, the detection was repeated by using Southern blotting (28) after running 15 μl of plasmid preparation in a 0.8% agarose gel. Hybridizations after Southern blotting were done as described above for dot blots. The presence of the genes was used as a proxy for the proteins they encode.

Adherence of STEC on HEp-2 cell cultures. HEp-2 cell adherence assays were performed according to standard protocols (7, 20). Briefly, 5×10^4 HEp-2 cells were incubated in 400 μl of Eagle's minimum essential medium (EMEM) with antibiotic and 10% fetal calf serum (FCS) in each well of an eight-well permanox culture slide (Nalge Nunc International Naperville, Il.) at 37°C in a 5% CO₂ atmosphere. The cells were washed three times with phosphate-buffered saline (PBS; pH 7.2) before use. The STEC strains to be tested were cultivated under aerobic conditions overnight at 37°C in Luria-Bertani broth and subcultured in EMEM with 10% FCS and 1 mM CaCl₂ at 37°C overnight in a 5% CO₂ atmosphere. Approximately 4×10^6 bacteria were inoculated in 300 μl of EMEM with 10% FCS and 1% D-mannose in each well of the culture slides and incubated for 3 h at 37°C in a 5% CO₂ atmosphere. The wells were washed three times with PBS, and the cells were incubated for 3 more hours under the same conditions. The slides were then washed three times with PBS, and the cells were stained with the Diff-quick staining kit (Dade Diagnostics Inc. Aguada, Puerto Rico) following the instructions of the manufacturer. A total of 200 cells were examined under the microscope, and the cells with more than 10 adherent bacteria per cell were counted. Strain 6-264 (O157:H7) was used as a positive control for each batch of tests. To control for day-to-day variation, all the results were reported in proportion to the positive control. To control for within-day variations, all tests were done in duplicate, and the results are expressed as the average of the duplicates.

Statistical analysis. All analyses were performed with SAS for Windows version 6.12 (SAS Institute Inc., Cary, N.C.). For the analysis of associations between virulence factors and isolates of serotypes associated with humans or of serotypes known to be involved in severe disease (set 1), univariate analysis with chi-square tests (34) and multivariate analysis with logistic regression, including a backward-elimination procedure (threshold of 5% significance), were used (14). Associations between covariates were analyzed with McNemar's association

tests (34). Reproducibility of the HEp-2 cell adherence assay was assessed by calculating an intracluster correlation coefficient (34) with a generalized linear model. To test for potential interactions between *eae* and the genes of the other factors in the logistic regression model, a manual forward procedure was used with a threshold of 5% significance (statistical interactions are present when two explanatory variables do not act independently on a response variable, thus suggesting the presence of synergism or antagonism at the biological level). The same procedures were used for the analysis of associations between virulence factors and severity of disease in isolates of human origin (set 2). However, for the latter analysis, the serotype variables were forced into the model, the level of adherence on HEp-2 was also included, and the *eae* variable was not used because all the isolates under study were positive for this characteristic. Finally, the same approach was used for the comparison of virulence factors and adherence level in STEC isolates of six major STEC serotypes of human origin versus those of bovine origin and of isolates from severe or less severe human disease versus those of bovine origin (set 3).

RESULTS

Homogeneity of virulence factors within serotypes (set 1). All the isolates within a serotype were identical in terms of presence or absence of the *eae* gene for the 65 serotypes with more than one isolate in set 1. Within these 65 serotypes, the *ehxA* and *espP* genes were consistently present or absent in 54 and 52 serotypes, respectively. The variability in terms of Shiga toxin was slightly higher, with 43 and 41 serotypes with homogeneous patterns for *stx₁* and *stx₂*, respectively. A strong association was present between *eae* and *ehxA* in the McNemar test ($P < 0.0001$; odds ratio [OR] = 9.3).

Associations between virulence factors and isolates of serotypes reported in humans (set 1). The distribution of the genes for the virulence factors under study in the different categories of set 1 is presented in Table 2. The results of the chi-square tests for the comparison of isolates from serotypes found in humans and those from serotypes not found in humans are reported in the fourth column of Table 2. When modeling the associations among the five virulence factors encoded by *ehxA*, *espP*, *eae*, *stx₁*, and *stx₂* and presence in humans with a logistic regression model, only *eae* and *stx₂* appeared as significant variables. This was the case in both a full logistic model comprising all the virulence factors as independent variables and in a reduced model resulting from the backward-elimination procedure. The only significant interaction between intimin and the other virulence factors of STEC at the 5% level was between Eae and Stx2. The coefficients, corresponding ORs, and *P* values for the two models with and without interaction are reported in Table 3 and 4.

Associations between virulence factors and isolates of serotypes reported in severe human disease (set 1). The proce-

TABLE 3. Coefficients, *P* values, and ORs for the logistic regression models of the association between virulence factors and STEC isolates from serotypes reported in humans[a]

Gene	Model 1			Model 2		
	β	*P*	OR	β	*P*	OR
ehxA	–	–	–	–	–	–
espP	–	–	–	–	–	–
eae	1.08	0.0008	2.93	0.55	0.1719	1.74
stx₁	–	–	–	–	–	–
stx₂	1.23	0.0001	3.41	0.86	0.0114	2.36
*eae*stx₂*	–	–	–	1.72	0.0473	NA

[a] The first model was obtained by using a backward-elimination procedure with a threshold of 5% significance. The second model was derived from the first one by using a forward procedure with a threshold of 5% significance to detect significant two-way interaction terms. β, coefficient; *eae*stx₂*, interaction between *eae* and *stx₂*; –, variables not significant at the 5% level and not included in the final models; NA, not applicable.

TABLE 4. Detailed ORs for the logistic regression model of the
association between virulence factors and STEC isolates
from serotypes reported in humans, including
the $eae*stx_2$ interaction (set 1)[a]

Comparison	OR
eae positive stx_2 negative vs eae negative stx_2 negative	1.74
eae negative stx_2 positive vs eae negative stx_2 negative	2.36
eae positive stx_2 positive vs eae positive stx_2 negative	13.20
eae positive stx_2 positive vs eae negative stx_2 positive	9.68
eae positive stx_2 positive vs eae negative stx_2 negative	22.87

[a] eae positive, isolate carrying the eae gene; eae negative, isolate lacking the eae gene; stx_2 positive, isolate carrying the stx_2 gene; stx_2 negative, isolate lacking the stx_2 gene. These results indicate, for instance, that an eae-positive and stx_2-positive STEC isolate is 13.2 times more likely to be from a serotype previously reported in humans than an eae-positive but stx_2-negative isolate.

dures described above were also used to compare isolates from serotypes found in severe human disease with those from other serotypes. The results of the chi-square tests for this comparison are reported in the last column of Table 2. The logistic regression analysis of these data resulted in a model similar to the previous one, with only eae (coefficient = 1.67; P value = 0.0001; OR = 5.33) and stx_2 (coefficient = 1.58; P value = 0.0001; OR = 4.86) significantly associated with isolates from serotypes found in severe disease. However, there was no evidence to suggest an interaction between intimin and the toxins.

Reproducibility of the HEp-2 cell adherence assay. Based on repeated trials of the HEp-2 cell adherence assays with a set of 15 isolates representing a broad range of adherence levels (0 to 1.4 in proportion to the positive control), an intracluster correlation coefficient of 0.89 was obtained. This result shows that 89% of the variability observed in the HEp-2 cell adherence assays is due to the strains and that only 11% of the variability is due to experimental error.

Associations between virulence factors of human STEC isolates from eight major serotypes and disease severity (set 2). The distribution of the genes for the virulence factors under study in the different categories of isolates from set 2 and the corresponding P values for the chi-square tests are presented in Table 5. Among the variables tested (serotype, $ehxA$, $espP$, stx_1 and stx_2, and level of adherence on HEp-2 cell cultures), stx_1 was associated with uncomplicated diarrhea and healthy individuals in the univariate analysis (Table 5) and stx_2 was significantly associated with severe disease in both the univariate analysis and the multivariate logistic regression models (coefficient = 1.60; P = 0.0038; OR = 4.95). There was no

evidence ($P > 0.05$) to suggest an interaction between the level of adherence on cell cultures and the toxins.

Comparison of distribution of virulence factors between isolates of human and bovine origin among six common STEC serotypes (set 3). The distribution of the genes for the virulence factors under study and the level of adherence on HEp-2 cell cultures are presented in Table 6. Univariate analysis with chi-square tests suggests a clear association between stx_1 and bovine isolates compared to that with human isolates in general (P value < 0.008). This crude analysis also suggests the same association when comparing isolates of bovine origin with those from humans with severe disease. In addition, STEC isolates from patients with uncomplicated diarrhea and healthy individuals seem to adhere significantly better to HEp-2 cells than do isolates from cattle. Logistic regression confirms the association between stx_1 and bovine isolates, when compared to human isolates in general, to isolates from patients with severe STEC-associated disease, or to isolates from patients with uncomplicated diarrhea and healthy individuals (Table 7). Logistic regression analysis shows similar associations for $espP$ and a significantly higher level of adherence on HEp-2 cells for STEC from humans in general, and particularly for STEC from patients with uncomplicated diarrhea and healthy individuals when compared to bovine isolates (Table 7). Finally, the logistic regression models also suggest a lower prevalence of stx_2 among isolates from patients with simple diarrhea and healthy individuals than among those from cattle.

DISCUSSION

Among over 100 serotypes that have been recovered from humans, serotypes O157:H7 and O157:H− clearly represent the majority of isolates associated with disease. However, STEC organisms of many other serotypes have been isolated from patients with HUS and HC with variable frequencies. The differences in frequencies may be partially related to reagent availability and methodological bias in the detection of STEC (9). However, previous studies have also shown a large spectrum of variability in virulence factor makeup in STEC populations, and many researchers have attempted to correlate the presence of specific recognized or putative virulence factors with disease or severity of disease (10, 12, 17, 22, 24, 29, 30, 32, 33, 38, 39). The main conclusion of these previous investigations has been that no single factor is responsible for the virulence of STEC. In all these studies, the role of each factor has been analyzed separately, without accounting for linkages between virulence factors. This simple approach may bias estimates of the role of putative virulence factors in disease pathogenesis by not correcting for the con-

TABLE 5. Overall distribution of virulence factors in 112 human STEC isolates of serotypes O26:H11, O26:H−, O103:H2, O111:H8,
O111:H−, O145:H−, O157:H7, and O157:H− isolated from individuals with severe disease
or with either uncomplicated diarrhea or no symptoms (set 2)

Gene or characteristic	Distribution[a]		
	Total ($n = 112$)	HUS and HC ($n = 54$)	D and NS ($n = 58$)
$ehxA$	101 (90.2)	50 (92.6; 0.4075)	51 (87.9)
$espP$	86 (76.8)	42 (77.8; 0.8104)	44 (75.9)
eae	112 (100.0)	54 (100.0; 1.000)	58 (100.0)
stx_1	75 (67.0)	29 (53.7; 0.0040)	46 (79.3)
stx_2	60 (53.6)	39 (72.2; 0.0001)	21 (36.2)
HEp-2[b]	1.144 (SD = 0.810)	1.056 (SD = 0.696; 0.2662)	1.227 (SD = 0.901)

[a] BD, bloody diarrhea; D, uncomplicated diarrhea; NS, no symptoms. The first number in parentheses indicates the percentage of the total category positive for the characteristic. The second number in parentheses for the third column indicates the P value for a chi-square test comparing isolates from severely diseased patients with those from patients with uncomplicated diarrhea and healthy individuals.
[b] HEp-2, level of adherence on HEp-2 cell cultures measured in proportion to a positive control.

TABLE 6. Overall distribution of virulence factors in 168 human and bovine STEC isolates of serotypes O26:H11, O103:H2, O111:H8, O111:H−, O145:H−, and O157:H7 (set 3)

Gene or characteristic	Distribution[a]				
	Overall ($n = 168$)	Bovine ($n = 77$)	Human ($n = 91$)	HUS + BD ($n = 46$)	D + NS ($n = 45$)
$ehxA$	158 (94.0)	73 (94.8)	85 (93.4; 0.7026)	45 (97.8; 0.4117)	40 (88.9; 0.2277)
$espP$	140 (83.3)	69 (89.6)	71 (78.0; 0.0446)	37 (80.4; 0.0224)	34 (75.6; 0.0389)
eae	168 (100.0)	77 (100.0)	91 (100.0; 1.0000)	46 (100; 1.0000)	45 (100; 1.0000)
stx_1	129 (76.8)	67 (87.0)	62 (68.0; 0.0039)	26 (56.5; 0.0001)	36 (80.0; 0.3027)
stx_2	83 (49.4)	37 (48.1)	46 (50.5; 0.7470)	33 (71.7; 0.0103)	13 (28.9; 0.0378)
HEp-2[b]	0.991 (SD = 0.741)	0.847 (SD = 0.557)	1.112 (SD = 0.852; 0.0203)	1.004 (SD = 0.682; 0.1667)	1.223 (SD = 0.992; 0.0083)

[a] The first number in parentheses indicates the percentage of the total category positive for the respective characteristic. The second number in parentheses for the last three columns indicates the P value for the comparisons with the isolates of bovine origin in chi-square tests.

[b] HEp-2, level of adherence on HEp-2 cell cultures measured in proportion to a positive control.

founding effect of other virulence factors and by neglecting joint effects as observed in the case of synergistic mechanisms.

The eae gene and the entire LEE can be spread horizontally in STEC populations. However, this event seems to be rare, and the presence of the LEE is strongly associated with particular STEC lineages (4). The $ehxA$ and the $espP$ genes are carried on the same plasmid (5) and are therefore physically linked. Recent work in our laboratory suggests some associations between the LEE, the EHEC hemolysin plasmid, and the hemolysin itself (4). Previous studies have shown a certain degree of homogeneity for the presence of virulence factors within STEC serotypes (12, 29, 30), and the results of the present study confirm this observation. This is also true, although at a slightly lower level, for the phage-encoded (35) Shiga toxin genes. Analysis of $E. coli$ populations by use of multilocus enzyme electrophoresis (6) has shown that serotype is a good marker for evolutionary lineages and is therefore also likely to be a good marker for the genetic background of STEC in terms of unknown virulence factors involved in the pathogenesis of STEC-associated diseases. Altogether, these data strongly support the approach taken in the present study, in which we tried to control for the confounding effects of the above-described genetic links among virulence factors and between virulence and serotype, an approach not used in previous works.

The first part of our study examined the association between the virulence factors of STEC and isolates from serotypes found in humans or in severe disease. Data on the exact origins of STEC isolates received in microbiological laboratories and reference collections are often very sparse, in particular with regard to clinical information. To overcome this limiting factor, we chose to use for the first part of the study a classification of isolates based on serotypes and their respective associations with humans as stated in the literature. Due to a lack of exhaustive descriptions and reporting in the literature, this approach may be subject to misclassification. It is expected that if this type of misclassification occurs, it will tend to decrease the significance of potential associations. Therefore, our approach may tend to ignore some weak but otherwise significant associations.

We observed no major difference in the frequency of $ehxA$ and $espP$ between isolates from serotypes found in humans and those not found in humans (Table 2). However, eae and stx_2 were significantly more frequent in isolates from serotypes found in humans, and this association was even more significant when we compared isolates from serotypes clearly associated with severe disease to isolates from other serotypes. The reverse is true for stx_1, which seems to be found more frequently among isolates from serotypes not found in humans than among those associated with humans. A significant difference in $ehxA$ frequency was observed between isolates from serotypes specifically associated with severe disease and those

that are not. This is not the case for $espP$. These crude data suggest an association of eae and stx_2 with isolates of serotypes found in humans and possibly of eae, stx_2, and $ehxA$ with severity of disease (Table 2). Our results are in agreement with those of previous studies showing that $ehxA$ (29, 32), eae (24), and stx_2 (22, 33, 37) are found more frequently in STEC isolates from patients with severe disease than in other STEC populations and that stx_1 may be associated with some STEC isolates of bovine origin (18, 40). However, in our multivariate analysis, only eae and stx_2 were significantly associated with isolates from serotypes found in humans or with isolates from serotypes implicated in severe human disease. This suggests that most of the crude association of EHEC hemolysin with severe disease could be due to confounding effects of the major virulence factor intimin (Fig. 2A). Alternatively, collinearity between eae and $ehxA$ in our model may obscure the true relationship between $ehxA$ and human STEC isolates or disease (Fig. 2B). Thus, as has been shown by others (2, 29, 31, 32), our results confirm that EHEC hemolysin may represent an interesting virulence marker for STEC involved in severe human disease. How-

TABLE 7. Coefficients, P values, and ORs in logistic regression models describing associations between STEC virulence factors and origin of STEC isolates (human with severe disease or uncomplicated diarrhea and healthy individuals versus bovine)[a]

Gene or characteristic	Model 1			Model 2			Model 3		
	β	P	OR	β	P	OR	β	P	OR
$ehxA$	−	−	−	−	−	−	−	−	−
$espP$	−1.56	0.0094	0.21	−1.43	0.035	0.14	−1.60	0.0282	0.20
stx_1	−1.78	0.0006	0.17	−1.94	0.0006	0.24	−2.20	0.0046	0.11
stx_2	−	−	−	−	−	−	−1.88	0.0435	0.15
HEp-2	0.95	0.0047	2.59	−	−	−	1.22	0.0045	3.39

[a] Model 1, logistic regression model obtained by backward-elimination procedure and describing associations between STEC virulence factors and human isolates in comparison to bovine isolates; model 2, logistic regression model obtained by backward-elimination procedure and describing associations between virulence factors and isolates from severe disease in comparison to bovine isolates; model 3, logistic regression model obtained by backward-elimination procedure and describing the association between virulence factors and isolates from patients with uncomplicated diarrhea and healthy individuals in comparison to bovine isolates. The backward-elimination procedure had a threshold of 5% significance. HEp-2, level of adherence on HEp-2 cell cultures measured in proportion to a positive control; β, coefficient; −, variables not significant at the 5% level and not included in the model. The results indicate, for instance, that when all the other factors are kept constant, an $espP$-positive isolate had a five times less likely (equivalent to 0.20 times more likely) to originate from a healthy human or a human with simple diarrhea than from cattle (model 3). Similarly, for each increase of 100% in the adherence level (in comparison to the positive control), an isolate is 2.59 times more likely to originate from humans than from cattle (model 1).
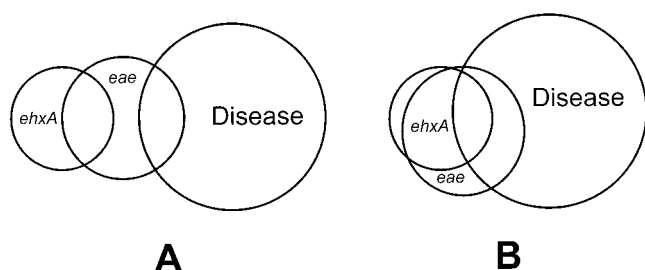
FIG. 2. Conceptual models of associations between *ehxA*, *eae*, and disease resulting in statistical association between *ehxA* and disease in univariate analysis but not in multivariate analysis (for reasons of simplicity, *stx*$_2$ was not included in these graphical representations). (A) Confounding effect of *eae*, i.e., strong association of *eae* with disease, no direct association between *ehxA* and disease, and strong association of *ehxA* with *eae*. (B) Collinearity of *eae* and *ehxA*, i.e., strong association of *eae* with disease, strong association of *ehxA* with disease, and strong association of *ehxA* with *eae*.

ever, our results also suggest that the role that EHEC hemolysin plays in the virulence of STEC may not be a major one, and therefore a reevaluation of its involvement in pathogenesis of severe disease due to STEC is warranted. Experimental work on animal and in vitro models is needed to clarify this point.

Adherence of STEC on enterocytes may be a necessary step for persistent colonization of the human intestine and for an efficient local delivery of toxins, allowing a significant absorption of Shiga toxins in or through enterocytes and more severe effects on the organism than would have occurred without adherence. It is therefore not surprising that our analysis shows an association between *eae* and isolates from serotypes found in humans and, moreover, one of our logistic regression models also suggests a possible interaction between *eae* and *stx*$_2$. It would be of interest, therefore, to confirm this finding on a larger collection of STEC with precisely defined origins.

The second part of our study concentrated on a few serotypes frequently associated with disease and evaluated the association of virulence factors within these serotypes with the severity of disease. Our results show a high prevalence of *eae* and *ehxA* in STEC isolates of these serotypes regardless of disease severity (Table 5). This confirms the observation made in the first part of the study suggesting an important role of intimin in strains from serotypes involved in disease and an association between the *eae* and *ehxA* genes in STEC populations. One observes a striking difference in prevalence of *stx*$_1$ and *stx*$_2$ between isolates from patients with severe disease and isolates from patients with simple diarrhea and healthy individuals. When we perform a logistic regression analysis (forcing the serotypes into the model), our results show a strong association between *stx*$_2$ and severe disease: an *stx*$_2$-positive isolate is approximately five times more likely to be associated with severe disease than an *stx*$_2$-negative isolate of the same serotype. In view of the results of the first part of the study, this conclusion is not surprising, and it fits with suggestions made by others using animal models (8, 36, 39) or less extensive epidemiological studies based on serogroup O157 only (22, 33, 37). No other factor reaches a significant level of association with severe disease in the logistic regression analysis. Interestingly, our full logistic regression model suggests a positive but statistically nonsignificant association between EHEC hemolysin and severity of disease (data not shown). Due to the high prevalence of EHEC hemolysin and low diversity in the population, only the analysis of a much larger number of isolates would allow us to confirm this association and to obtain a valid estimate of its coefficient. However, the results of the first part of this study suggest that this coefficient would probably be

relatively low. The observed level of STEC adherence on HEp-2 cell cultures did not show any significant association with severity of disease in the univariate or the multivariate logistic regression analysis for this part of the study.

The third part of our study used STEC isolates of six major serotypes frequently involved in disease to examine if STEC isolates of these serotypes isolated from humans may form a different population than those from the bovine STEC reservoir. Trends visible in the univariate analysis (Table 6) are confirmed by multivariate analysis (Table 7) and show a significant association of *stx*$_1$ and *espP* with bovine STEC populations of these serotypes. They also show that human isolates of these *eae*-positive serotypes adhere more strongly on HEp-2 cell cultures than those from cattle. This fact is particularly marked in the case of isolates from patients with diarrhea or from healthy carriers and suggests that increased adherence on epithelial cells may play a role in the pathogenesis of STEC-associated diarrhea. Our results from cell cultures are in agreement with another report (38) suggesting that adherence may be a more important factor in STEC-associated diarrhea than Shiga toxins. However, our results should be confirmed with other cell lines more representative of polarized enterocytes (42) or in more complex systems, like the recently described adherence tests on organ cultures (13). These models may be more relevant for assessment of the adherence of STEC. They may better mimic the in vivo conditions encountered by STEC in the human bowel, thus allowing the bacteria to fully express characteristics only poorly expressed on HEp-2 cell cultures.

In conclusion, our results formally show that intimin and Stx2 are the virulence factors of STEC that are most strongly associated with disease in humans, and particularly with severe disease. These results suggest that STEC strains carrying the *eae* and *stx*$_2$ genes should be the main targets of preventive and therapeutic measures. We could not detect any significant association of the newly described EspP protease with disease in humans. In contrast with previous studies using univariate analysis, the present work using multivariate analysis did not show any significant association between EHEC hemolysin and disease. This may be due either to a true lack of biological significance of EHEC hemolysin in the pathogenesis of STEC-associated diseases or to collinearity problems in multivariate modeling. The latter point clearly needs further clarification. Our results show that distribution of virulence factors and adherence levels differ between human and animal populations of the same serotypes. Thus, our results strongly suggest that STEC isolates from humans form a different population than those found in the bovine reservoir or that they are only a subpopulation of the latter.

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